(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 January 2002 (24.01.2002)

PCT

(10) International Publication Number
WO 02/06450 A1

(51) International Patent Classification[7]: C12N 5/10, C12Q 1/02

(21) International Application Number: PCT/EP01/08165

(22) International Filing Date: 13 July 2001 (13.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
00115489.7 18 July 2000 (18.07.2000) EP

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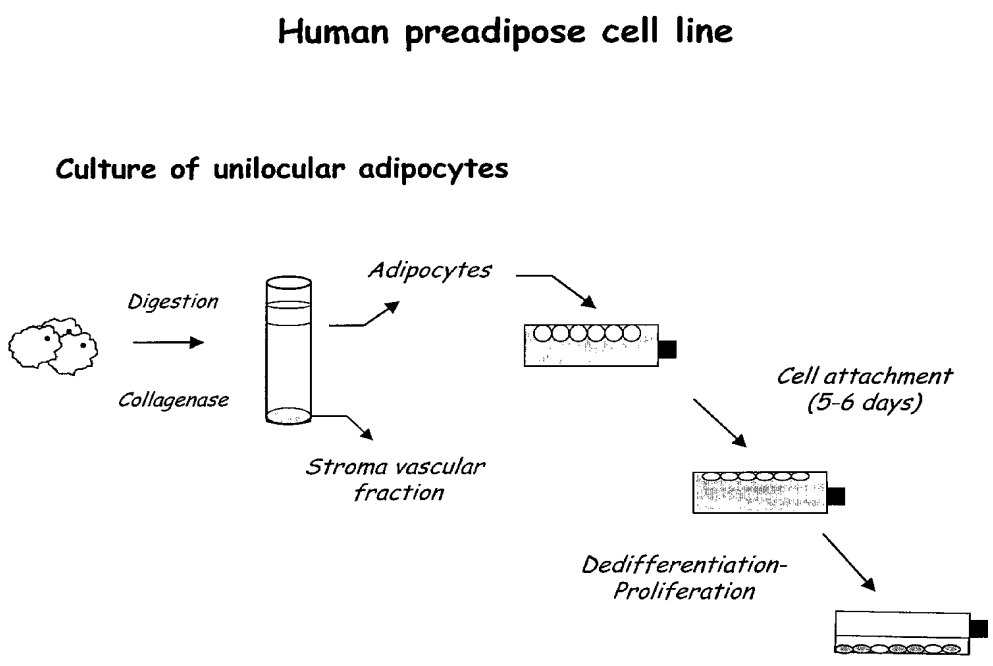
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*

*[Continued on next page]*

(54) Title: PRE-ADIPOSE CELL LINES




(57) Abstract: This invention relates to new human pre-adipose cell lines capable to differentiate to adipose cells. In particular, the present invention pertains to adipocyte cell lines derived from white adipose tissue and their use in developing drugs, food ingredients and supplements against obesity, diabetes and cardiovascular diseases.

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# Pre-adipose Cell Lines

This invention relates to human pre-adipose cell lines capable to differentiate into adipose cells. In particular, the present invention pertains to pre-adipose cell lines derived from white adipose tissue and their use in developing drugs, food ingredients and supplements against obesity, diabetes and cardio-vascular diseases.

Obesity has been declared a public health hazard by the National Institutes of Health. Obesity may be defined as the presence of excess adipose tissue which affects about 30 to 50 % of the North American population and a substantial percentage of the population worldwide. The effects of obesity, e.g. non-insulin dependent diabetes, coronary artery disease, and hypertension, are estimated to have resulted in $45.8 billion in direct costs and an additional $23 billion in indirect costs from e.g. missed work.

It is held that excessive ingestion of fat and carbohydrate induces obesity and hyperlipidemia and even develops hypertension and arteriosclerosis ultimately. The desirability of repressing the absorption of fat and carbohydrate and diminishing the accumulation of fat has, therefore, been finding enthusiastic recognition. Infants, on exposure to excessive caloric intake, suffer increase of adipocytes and assume the state which may well be called potential obesity. By this reason, it has been reported that the repression of the increase of the number of adipocytes particularly in infants results directly in the prevention of the obesity and the cardiovascular diseases which may well be called complications of obesity in children and consequently in adults.

To combat this evident health problem, both prophylactic and therapeutic approaches are necessary. For prophylactic purposes, it would be useful to be able to predict and measure a person's propensity or susceptibility to obesity. For therapeutic purposes, a means for

interfering with the development or differentiation of adipocytes (fat cells) would be of great benefit. None of these desired objectives has been achieved so far, which is mainly due to that little is known about the regulation and control of the development of adipose tissue, e.g. the proliferation and differentiation of adipocytes precursors.

The identification of means or substances, respectively, controlling such proliferation and differentiation is very important for understanding normal adipose tissue development and for designing approaches for controlling abnormal states of adipose tissue development such as obesity.

To determine whether a substance is involved in the physiological regulation of adipose tissue development, investigations may be carried out if adipocytes or precursors thereof are responsive to this factor and ultimately if this factor can efficiently and specifically modulate adipose tissue development *in vivo*. Appropriate studies could not be performed so far, which fact is mainly due to a lack of suitable tissue or cells derived from human adipose tissue exhibiting properties of normal adipocytes from living tissue and being capable to be cultured for a prolonged period *in vitro* so as to carry out the experiments.

Fat cells or adipocytes (adipose cells) the main cell population of fat tissue represent a principal storage depot for triglycerides, and are deemed to be endocrine cells. Adipose tissue provides an energy storage reserve for the body in the form of triglycerides and this tissue can release free fatty acids when caloric intake falls below metabolic needs. In response to increased dietary intake, the body will normally automatically increase energy expenditure through activity to maintain an energy balance. Energy can also be released as heat. There are common energy regulation pathways that balance dietary intake with metabolic activity largely mediated through the hypothalamus. It is now also apparent that the adipocytes play an active role in this process and likely produce molecules that serve to feed back and effect regulation of triglyceride metabolism. Furthermore, adipocytes are able to secrete hormones which modulate key functions in peripheric or central organs. The best example being the leptin secreted by adipocytes and regulating energy metabolism and satiety via receptors located in hypothalamus. It would therefore be of great interest to be capable to find and

investigate such molecules.

There are essentially two types of adipose tissue, brown and white, which carry out quite different roles in the body. White adipose is designed to store excess caloric intake while brown adipose tissue uses a unique system to syphon off excess calories and use it to generate body heat. However, white adipocytes were shown to express uncoupling proteins involving the control of thermogenesis. Since only white adipose tissue subsists in adult humans, thermogenesis induced by white adipose may increase energy expenditure.

An object of the present invention resides therefore in providing means to further investigate the role of white adipose tissue in the body.

Another object of the invention is to provide means that allows an investigation of the effect of novel drugs or food ingredients on white adipose tissue.

The above object has been solved by providing novel pre-adipose cell lines derived from white adipose cells that have the capability to differentiate into mature white adipose cells, while exhibit essentially the same cellular properties as do normal white adipose cells.

In the figures,

Fig. 1 schematically shows how the pre-adipose cells were obtained;

Fig. 2 shows the expression of the SV40 large T-antigen in the various immortalized clones analyzed by RT-PCR and immuno-fluorescence;

Fig. 3 shows the cell differentiation under different adipogenic conditions;

Fig. 4 shows the results of experiments investigating the expression pattern of different markers of adipocyte differentiation in the cell line as compared with human adipose tissue.

Cell lines have played an important role in the development of molecular and cellular biology, particularly in the elucidation of intracellular activities, the effects of extracellular molecules and cell-cell interactions. Cell lines are normally established stepwise by explantation of tissue containing a heterogeneous cell population, separation of the cells, isolation of a cell clone of interest and culturing the cell clone so that the total cell number increases over several generations and the population is uniform in its lineage. Yet these cells will survive only a limited number of passages in an *in vitro* culture before they start to senesce.

Cells that can be cultured essentially continuously are known as so called immortalized cells. Immortalized cells have many advantages over non-immortalized cells because they can be cultured to provide a large number of an uniform cell population. Normally, immortalized cell lines are prepared by means of a recombinant virus or a retrovirus, which method does, however, result in the cells thus immortalized, being somewhat unbalanced in their cellular functions. To this end immortalized cell lines most often substantially differ from the cells they are derived from in that they exhibit a different metabolic pattern not producing enzymatic or structural polypeptides normally found in said type of cell. What is more, also the potential for differentiation is strongly influenced thereby. Consequently, many cell types have remained difficult to isolate and to culture continuously.

The present invention now provides for novel immortalized cell lines capable to differentiate to mature to white adipocytes, which white adipocytes will exhibit essentially the same morphological pattern as e.g. exhibited by their polypeptide expression or cellular appearance as do white adipocytes obtained from living tissue.

The pre-adipose cell lines of the present invention may be differentiated to mature white adipocytes with compounds known in the art, such as insuline, triiodothyronine, dexamethasone and activators of peroxisome proliferator-activated receptors (PPAR). The white adipocytes thus obtained will then essentially show the same metabolic markers as do normal white adipose cells, such as enzymes involved in triglyceride synthesis, e.g. lipoprotein lipase and fatty acid synthase, adipocyte fatty acid binding protein, leptin, adipsin,

adiponectin, peroxisone proliferator-activated receptors γ (PPARγ) and the hormone sensitive lipase. The white adipocytes may be kept in culture for at least 12 passages, preferably at least 20 passages, more preferably at least 30 passages and most preferably at least 50 passages.

According to a preferred embodiment the cell line is any of the cell lines deposited according to the Budapest Treaty with the Institute Pasteur on July 13th, 2000, receiving the Deposit no CNCM I-2520 or the cell line deposited on April 27th, 2001, receiving the deposit no CNCM I 2663.

The method for preparing the cell lines of the present invention comprises the following steps (a) to (e).

During step (a) white adipose cells are isolated in vitro from an appropriate adipose tissue from a human donor. The primary tissue obtained from the donor is first treated such that the adipocytes are separated from other cells present, such as e.g. by treatment of the tissue sample with a solution containing collagenase and separating adipocytes from the other cells by successive filtrations followed by a centrifugation.

In the subsequent step (b) the adipose cells obtained in step (a) are de-differentiated, which may be effected by using e.g. the so called "ceiling culture method" previously described for rodent adipocytes (Sugihara H., Yonemitsu N., Miyabara S., Yun K. 1986. Primary culture of unilocular fat cells: characteristics of growth in vitro and changes in differentiation properties. Differentiation, 31: 42-49). According to this method the adipocytes are transferred into a culture flask, that is filled to the top with culture medium and positioned upside down. After six days under these conditions, adipocytes de-differentiated spontaneously into fibroblast-like cells deprived of lipid droplets. The resulting de-differentiated adipocytes are then proliferated under the same culture condition as above to obtain actively growing preadipocytes.

In the next step (c) the preadipocytes thus obtained are immortalized. This may be achieved

by infecting cells with a recombinant vector, such as with a recombinant plasmid, a recombinant virus or a retrovirus, e.g. a recombinant retroviral vector carrying the large T antigen gene of SV40 virus (Simian Virus) or the E6 or E7 genes of HPV virus (Human Papilloma Virus). According to an alternative method the recombinant vector harbors the telomerase reverse transcriptase (TERT) genes, which are preferably derived from the species the cells of which shall be immortalized. The gist of the latter method resides in essentially preventing a shortening of the chromosome's telomeres, an effect well known to be accompanied with senesceing. Telomere maintenance and cellular immortalization has been reported in epithelial cells in Kiyono et al, Nature **396** (1998), 84-88, which document is incorporated herein by way of reference.

A successive infection with SV40 virus (Simian Virus) or the E6 or E7 genes of HPV virus and the TERT gene represents also an alternative method for human preadipocyte immortalization.

In the subsequent step (d) a selection is made for cells positively immortalized, which may e.g. simply be performed by culturing cells obtained in step (c) for several passages or by testing the cells for genes of the vector used for the immortalization, such as for genes from the SV40 virus or genes from the HPV virus. This may be achieved by detecting the expression of the respective genes by means of antibodies or may involve an analysis via PCR-techniques. The expression of the telomerase reverse transcriptase genes is detected by measuring the telomerase activity determined by applying the Telomerase Repeat Amplification Protocol (TRAP).

In the subsequent step (e) the cells shown to be positively immortalized by the introduction of the vector are tested for their ability to differentiate into mature white adipose cells. To this end, the cell lines obtained under step (d) are treated with common differentiating agents, such as detectable by intracellular lipids staining. Lipids staining is commonly performed with the Oil-red-O method which specifically stains lipids.

The cell lines of the present invention may be used for different purposes, such as serving as

a means to investigate the role of controlling the regulation of lipid uptake and release by white adipocytes, or the identification of substance controlling the differentiation of pre-adipocytes into mature adipocytes, or for screening for compounds capable to control the expression of targets for obesity, diabetes and cardio-vascular diseases. Particularly, these cell lines may be helpful for the screening of factors able to regulate the release by adipocytes of compounds involved in the control energy metabolism such as leptin.

The following examples will illustrate the invention without limiting it to the specific embodiments mentioned.

Example 1

ADIPOCYTE EXTRACTION FROM HUMAN SUBCUTANEOUS ADIPOSE TISSUE

After surgery the biopsy of subcutaneous adipose tissue from an obese patient was maintained at room temperature with a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 media (v/v) (Gibco BRL) supplemented with 10% fetal calf serum (FCS) (Gibco BRL), 100 µg/ml streptomycine/penicilline and 2 mM glutamine. This medium was termed basic medium. The adipose tissue was minced into small pieces and digested 20 min at 37°C into 3 ml of digestion medium per gram of tissue. The digestion medium contained 2 mg/ml collagenase (Roche Biomedical) and 20 mg/ml bovine serum albumin (Sigma) dissolved into DMEM medium.

Digestion was stopped by the addition of an equivalent volume of FCS in order to get a 20 % FCS concentration. The digested fluid was filtered twice with filters of different porosity (250, 100 µm) and centrifuged 10 min at 1000 rpm. Adipocyte fraction was obtained as a thin and white floating layer which was removed and put into 30 ml phosphate buffer at 37°C. The suspension was centrifuged (10 min at 1000 rpm) in order to obtain an adipocyte fraction deprived of other contaminating cells.

**Example 2**

PRIMARY ADIPOCYTE DEDIFFERENTIATION AND PROLIFERATION

About $10^6$ adipocytes were incubated in a 25 $cm^2$ flask completely filled with DMEM mixed with Ham's F12 medium (v/v) supplemented with 20 % FCS, 10 mg/ml streptomycine/ penicilline and 2 mM glutamine. The flask was turned upside down in order to allow adipocytes to adhere to the top inner surface. After 8 days of culture in the appropriate environment (37°C, 90 % humidity) cells had attached and started to de-differentiate spontaneously into preadipocytes and to proliferate. The flask was then turned in the proper orientation and the medium was removed. 5 ml of the above described basic medium was added in the flask. After 2 days under these conditions cells were infected.

**Example 3**

CELL IMMORTALIZATION

1.Production of retrovirus

A recombinant retroviral vector carrying the large T antigen gene of SV40 virus (Simian Virus) or the E6/E7 genes of HPV virus (Human Papilloma Virus) or the human telomerase reverse transcriptase (hTERT)) were constructed by insertion, with standard recombinant DNA techniques, into the BamHI site of the pLHXSD retroviral vector (Stockschlaeder et al., Hum Gene Ther. **2** (1991), 33-39) containing the histidinol gene as selection marker.

Infectious recombinant virus particles were generated through transfection of the recombinant retroviral vector into the amphotropic packaging cell line Phoenix (Clontech), followed by co-culturing with the ecotropic packaging cell line, Psi2 (ATCC) to allow "ping-pong" infection to produce a high-titer virus (Lynch C, Miller D. 1991. Production of high helper virus-free retroviral vectors by cocultivation of packaging cells with different host racipes. J. Virol. 65: 3887-3890).

2. Infection of primary adipose cells

Infectious recombinant virus particles as prepared above were used to infect primary human de-differentiated adipose cells obtained according to example 2. The cells were incubated for 3 hours at 37°C (90 % humidity) with the recombinant virus in the presence of 20 µg/ml DEAE dextran. After the infection, the culture medium was changed with the basic medium.

Ten days after infection the first clones were picked up by aspiration and expanded separately. The expression of the SV40 T antigen or E7 genes in the different clones was determined by RT-PCR using

| | |
|---|---|
| SV40 T antigen: | 5'GGATTCAGTGGTGTATGACT; |
| | 5'AGGCACACTGTACTCATTCA; |
| E7: | 5'GGAGATACACCTACATTGCA; |
| | 5'GATGGGGCACACAATTCCTA |
| | (all purchased from by Microsynth); |

and immuno-staining utilizing mouse monoclonal antibodies directed against the human SV40 T antigen (Oncogene). The telomerase activity was determined by applying the Telomerase Repeat Amplification Protocol (TRAP) according to Kim et al., Science 266 (1994), 2011-2015.

## Example 4

**DIFFERENTIATION OF INFECTED CELLS**

7SV40 T antigen, E7, TERT or E7 and TERT positive cells as obtained in example 3 were incubated in the basic medium, as defined in example 1, to confluence at which stage an adipogenic cocktail was added to the medium which cocktail contained the basic medium and supplemented with 850 nM insulin; 10 µg/ml transferrin; 1 nM triiodothyronine; 500 µM fetuin, 33 µM panthotenic acid, 1 mM Hepes, 15 mM $NaHCO_3$ and 1µM dexamethasone and 1µM BRL49653, a PPARγ agonist. The cells were then continued to be incubated. At

day 10 after confluence cells started to exhibit an adipocytic phenotype with intracellular lipid droplets observed with a specific staining. This Oil-red-O staining was performed on fixed cells with 10 % Formaldehyde incubated 2 hours with the dye dissolved at in isopropanol. Cells were then washed with water and observed under microscope. Differentiated adipocytes appeared as cells filled with red cytoplasmic spots corresponding to the accumulation of lipid droplets.

**Example 5**

**EXPRESSION OF ADIPOCYTES IN DIFFERENTIATED IMMORTALIZED HUMAN PREADIPOSE CELLS**

Confluent immortalized human preadipose cells of the cell line CNCM I-2550 (as obtained in the preceding examples) were cultured in a serum-free chemically defined medium described in example 4.

At day 17 after confluence, cells were washed with Hank's balanced salt solution and RNA was extracted using the RNeasy Total RNA Purification System (Qiagen AG, Switzerland). Reverse transcription was performed with an input of 2 µg of total RNA using the 1st strand cDNA synthesis kit for RT-PCR (AMV; Roche Biomedical, Switzerland) with oligo $d(T)_{15}$ as primer. Primers used for the amplification of cDNA's of interest were synthesized by Mycrosynth (Windisch, Switzerland).

The sequences of the forward and reverse primers were:

| | |
|---|---|
| Actin | Forward 5'-GTTGCTATCCAGGCTGTG-3' |
| | reverse 5'-CATAGTCCGCCTAGAAGC-3' |
| Adipsine | forward 5'-TACAGCTGTCGGAGAAGG-3' |
| | reverse 5'-TTCTTGCGGTTGCCGCAAAC-3' |
| Adiponectin | forward 5'-GGGAGCTGTTCTACTGCTAT-3' |
| | reverse 5'-CTCCAATCCCACACTGAATG-3' |
| Fatty Acid Binding protein (aFABP) | forward 5'-GGTACCTGGAAACTTGTCTC-3' |
| | reverse 5'-AACTTCAGTCCAGGTCAACG-3' |
| Lipoprotein Lipase (LPL) | forward 5'-TTTCTCTGTATGGCACCGTG-3' |
| | reverse 5'-TTCACAAATACCGCAGGTGC-3' |
| Fatty Acid Synthase (FAS) | forward 5'-GGTCTTGAGAGATGGCTTGC-3' |
| | reverse 5'-CAGGTTGACAGCAGCCAAGT-3' |
| Peroxysome proliferator acivated recceptor δ (PPAR δ) | forward 5'-TCAACGACCAGGTTACCCTT-3' |
| | reverse 5'-CTTGATCCGCTGCATCATCT-3' |
| Peroxysome proliferator acivated recceptor γ (PPAR γ) | forward 5'-GTGCAGGAGATCACAGAGAT-3' |
| | reverse 5'-TTGCCAAGTCGCTGTCATCT-3' |

The PCR reaction was heated for 2 cycles to 98°C for 1 min, 60°C for 2 min and 72°C for 2 min and then cycled 28 times through a 1 min denaturation step at 94°C, a 1 min annealing step at 60°C and a 2 min extension step at 72°C in a DNA thermal cycler apparatus (Bioconcept, Allschwil, Switzerland).

Actin primers were included in the reaction as an internal control. PCR products (10 µl) were separated on a 2% agarose gel and visualized by ethidium bromide staining.

The results clearly indicate that the cell line investigated expresses the respective adipocyte's markers in a manner essentially identical to adipose tissue as such.

# Claims

1. A human pre-adipose cell line capable to mature to white adipose cells, exhibiting essentially cellular properties of normal white adipose cells.

2. The human pre-adipose cell line according to claim 1, wherein the adipose cells differentiated from said pre-adipose cell line exhibit a metabolic pattern of lipoprotein lipase, fatty acid synthase, adipocyte fatty acid binding protein, leptin, adipsin, adiponectin, peroxisone proliferator-activated receptors γ and β (PPARγ, β) and the hormone sensitive lipase essentially identical to normal adipose cells.

3. The human pre-adipose cell line which is CNCM I-2520 or CNCM I-2663.

4. A method for preparing a pre-adipose cell line according to any of the preceding claims which comprises the steps
   (a) separating cells from a human adipose tissue;
   (b) de-differentiating and proliferating the cells obtained in (a) to obtain a pre-adipocyte clone;
   (c) immortalizing a pre-adipose clone isolated under step (b);
   (d) selecting for immortalized cells, and
   (e) selecting for cells capable to differentiate into white adipose cells.

5. The method according to claim 4, wherein the immortalization step has been carried out with a recombinant vector, carrying the SV40 T antigen.

6. The method according to claim 4, wherein the immortalization step has been carried out with a recombinant vector, carrying the E7 genes of HPV virus

7. The method according to claim 4, wherein the immortalization step has been carried out by means of the telomerase method.

8. The method according to claim 4, wherein the immortalization step has been carried out with the association of a recombinant vector carrying the E7 genes of HPV virus and a virus carrying the telomerase reverse transcriptase gene.

9. Use of a cell line according to any of the claims 1 to 3 for the identification of a substance controlling the regulation of lipid uptake and release by human white adipocytes.

10. Use of a cell line according to any of the claims 1 to 3 for the identification of a substance controlling the differentiation of preadipocytes into mature adipoytes.

11. Use of a cell line according to any of the claims 1 to 3 for screening for compounds capable to control the expression of targets for obesity, cardio-vascular diseases and diabetes.

12. Use of a cell line according to any of the claims 1 to 3 for screening for compounds capable to regulate the secretion of any metabolites or hormones from human white adipocytes.

# Human preadipose cell line

## Culture of unilocular adipocytes

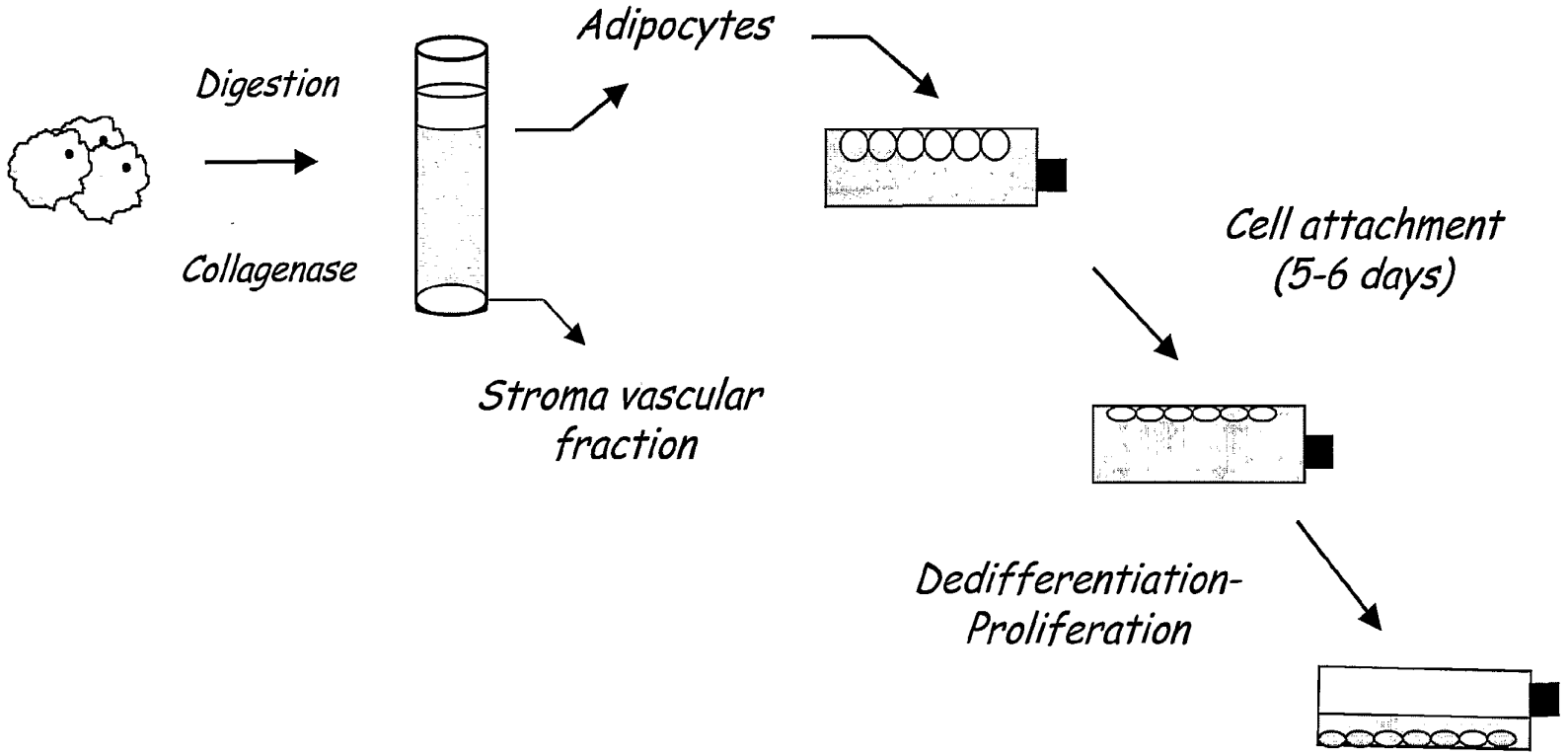
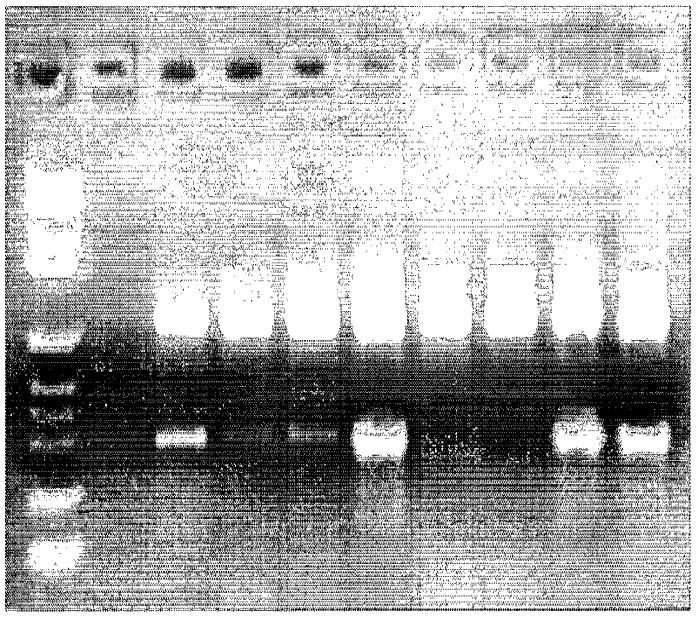



Fig.1

# Human preadipose cell line

## Expression of the T antigen

- mRNA:




- Protein (Immunostaining):

| | Positive cells |
|---|---|
| Visceral preadipocytes p.4 ( polyclonal population) | 20 % |
| Subcutaneous preadipocytes p. 5 ( polyclonal population) | 100 % |

Fig.2

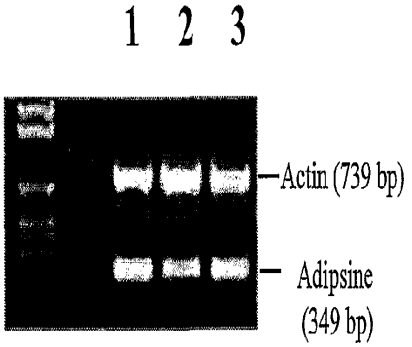
Fig.3

Human preadipose cell line

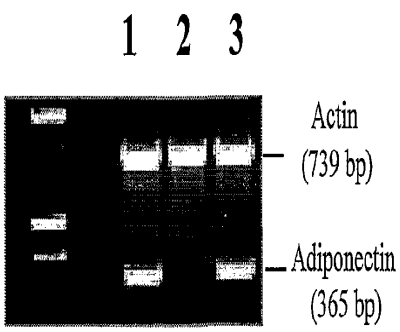
Cell differentiation under different adipogenic conditions

Subcutaneous
polyclonal pop. (p. 5)

Adipsine




Adiponectin



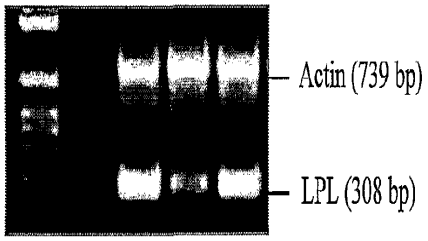

Conditions:

1- Human subcutaneous adipose tissue

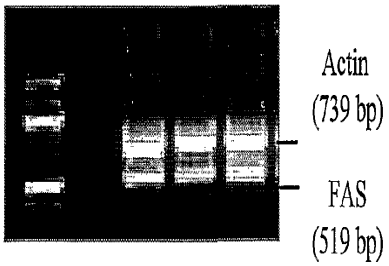
2- Differentiated immortalized human preadipose cells

3- Differentiated immortalized human preadipose cells with a PPARγ agonist

LPL




FAS




Abbreviation:

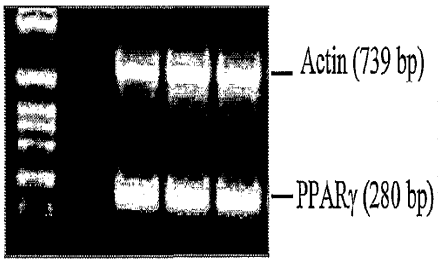
**aFABP:** adipocyte Fatty Acid Binding protein

**FAS:** Fatty Acid Synthase

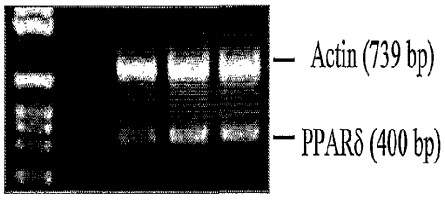
**LPL:** Lipoprotein lipase

**PPAR:** Peroxysome proliferator activated receptor

PPARγ




PPARδ




aFABP

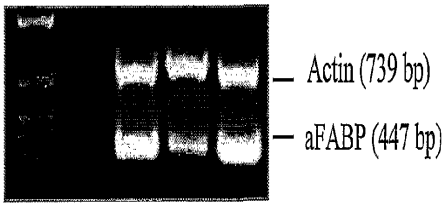



Fig. 4

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TRAITE DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES
AUX FINS DE LA PROCEDURE EN MATIERE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE :

**SOCIETE DES PRODUITS NESTLE SA**
**Patents Department**
**Avenue Nestlé 55**
**CH-1800 VEVEY**

NOM ET ADRESSE
DU DEPOSANT

RECEPISSE EN CAS DE DEPOT INITIAL,
délivré en vertu de la règle 7.1 par
l'AUTORITE DE DEPOT INTERNATIONALE
identifiée au bas de cette page

| I. IDENTIFICATION DU MICRO-ORGANISME | |
|---|---|
| Référence d'identification donnée par le DEPOSANT : **ADScSV40-C6** | Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE : **I - 2520** |

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

■ d'une description scientifique

■ d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le **13 juillet 2000** (date du dépôt initial) [1]

IV. RECEPTION D'UNE REQUETE EN CONVERSION

La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial)
et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)

V. AUTORITE DE DEPOT INTERNATIONALE

| | |
|---|---|
| Nom : **CNCM** Collection Nationale de Cultures de Microorganismes<br>Adresse : **INSTITUT PASTEUR** 28, Rue du Docteur Roux F-75724 PARIS CEDEX 15 | Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : **Georges WAGENER**<br>Date : **Paris, le 25 août 2000** |

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)

TRAITE DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES
AUX FINS DE LA PROCEDURE EN MATIERE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE :

RECEPISSE EN CAS DE DEPOT INITIAL,
délivré en vertu de la règle 7.1 par
l'AUTORITE DE DEPOT INTERNATIONALE
identifiée au bas de cette page

**SOCIETE DES PRODUITS NESTLE SA**
**Patents Department**
**Avenue Nestlé 55**
**CH-1800 VEVEY**

NOM ET ADRESSE
DU DEPOSANT

| I. IDENTIFICATION DU MICRO-ORGANISME | |
|---|---|
| Référence d'identification donnée par le DEPOSANT : **CHUB-S7** | Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE : **I - 2663** |

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

■ d'une description scientifique

■ d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le **27 avril 2001** (date du dépôt initial) [1]

IV. RECEPTION D'UNE REQUETE EN CONVERSION

La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial)
et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)

V. AUTORITE DE DEPOT INTERNATIONALE

| | |
|---|---|
| Nom : **CNCM** Collection Nationale de Cultures de Microorganismes<br>Adresse : **INSTITUT PASTEUR** 28, Rue du Docteur Roux F-75724 PARIS CEDEX 15 | Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : **Georges WAGENER**<br>Date : **Paris, le 10 septembre 2001** |

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)

# INTERNATIONAL SEARCH REPORT

Inte[illegible]nal Application No
PC[illegible]P 01/08165

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/10 C12Q1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 6 071 747 A (ZILBERFARB VLADIMIR ET AL) 6 June 2000 (2000-06-06) column 2, line 5 -column 5, line 16; example 1 | 1,2,4-12 |
| Y | --- | 3 |
| Y | SUGIHARA H.: "Primary cultures of unilocular fat cells: characteristics of growth in vitro and changes in differentiation properties" DIFFERENTIATION, vol. 31, 1996, pages 42-49, XP000990058 cited in the application the whole document | 3 |
| | --- -/-- | |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 December 2001 | 02/01/2002 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31–70) 340–2040, Tx. 31 651 epo nl,<br>Fax: (+31–70) 340–3016 | Fernandez y Branas,F |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/08165

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 00 24866 A (INST NAT SANTE RECH MED ;MARIE PIERRE (FR); LOMRI ABDERRAHIM (FR);) 4 May 2000 (2000-05-04) page 5, line 35 -page 6, line 34 page 12, line 32 - line 36 claims 1,5,9 | 1,2,7-12 |
| X | US 5 830 682 A (MOORE EMMA E) 3 November 1998 (1998-11-03) example 3 | 1,2,7-12 |
| X | US 5 827 740 A (PITTENGER MARK F) 27 October 1998 (1998-10-27) column 2, line 46 -column 3, line 21; examples 1-3 | 1,2,7-12 |
| X | KOLAROVA P. ET AL: "Conditional immortalization of white adipocytes using SV40 large T-antigen from the transgenic mouse" EUROPEAN JOURNAL OF CELL BIOLOGY, vol. 74, no. suppl 47, 1997, page 87 XP000990293 abstract | 1,2,7-12 |
| A | THOMAS MICHAEL ET AL: "Formation of functional tissue from transplanted adrenocortical cells expressing telomerase reverse transcriptase." NATURE BIOTECHNOLOGY, vol. 18, no. 1, January 2000 (2000-01), pages 39-42, XP002185530 ISSN: 1087-0156 the whole document | 7 |

# INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 01/08165

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| US 6071747 A | 06-06-2000 | FR 2733513 A1 | 31-10-1996 |
| | | CA 2219996 A1 | 31-10-1996 |
| | | EP 0830450 A1 | 25-03-1998 |
| | | WO 9634100 A1 | 31-10-1996 |
| | | JP 11505414 T | 21-05-1999 |
| WO 0024866 A | 04-05-2000 | WO 0024866 A2 | 04-05-2000 |
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| | | US 5683906 A | 04-11-1997 |
| | | AU 3586595 A | 27-03-1996 |
| | | CA 2199377 A1 | 14-03-1996 |
| | | EP 0804554 A1 | 05-11-1997 |
| | | JP 10505498 T | 02-06-1998 |
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